

The Role of E2F3 Phosphorylation in Rb-mediated Tumorigenesis and Embryonic Development

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in Molecular Genetics in the College of Biological Sciences at the Ohio State University

by

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Abstract

Defects in the regulation of cellular replication are some of the primary causes of cancer in humans. The E2F3 protein, a member of the E2F transcription factor family, acts to control progression past the G₁/S-checkpoint during cellular replication. One way that E2F3 is regulated is via phosphorylation. The phosphorylated form of this protein is often found in tumor cell lines and is thought to be linked to cancer. However, the exact pathways and interactions of this protein are not well characterized *in vivo*. The purpose of this study is to better understand how E2F3 phosphorylation controls the cell cycle *in vivo* and thus how it affects development as well as tumor incidence, growth, and metastases in mice.

The *retinoblastoma* gene, which promotes pituitary and thyroid cancer when one copy is knocked out, was combined with *E2F3* mutations mimicking either the phosphorylated (E2F3^{S390D}) or non-phosphorylated (E2F3^{S390A}) form of the protein. We observed tumor incidence and size, as well as metastases to other organs in these mice at 11 months of age. Our data shows that a lack of E2F3 phosphorylation increases thyroid cancer incidence in female mice. Interestingly, both phospho-mimicking and non-phosphorylatable mutants of E2F3 appear to decrease pituitary tumor size, but not incidence, compared with wild type E2F3.

Developmentally, mice lacking the *retinoblastoma* gene typically die between the embryonic ages of 13.5 and 15.5 days. We observed survival at E11.5 and E13.5 days in Rb-null mice combined with one of the aforementioned E2F3 mutations. We show that embryos with the phospho-mimicking mutant of E2F3 have decreased viability compared to wild-type and non-phosphorylatable mutants at E11.5.

Since phosphorylation is an alteration at the chemical level, understanding this pathway could lead to novel forms of chemotherapy that help fight cancer associated with E2F3.

Introduction

This study aims to look at the effects of E2F3 phosphorylation in the complete or partial absence of a functional *retinoblastoma* gene product. Retinoblastoma is a member of the Retinoblastoma protein family, which also includes the proteins p107 and p130. These proteins, often referred to as pocket proteins, regulate the effects of E2F transcription factors by selectively binding them and regulating how they interact with target DNA¹ (**Figure 1**). The Rb protein is regulated via phosphorylation²⁻³ in a cell-cycle dependent manner⁴⁻⁷. This regulation occurs through the actions of cyclin dependent kinases (Cdk's) and their associated cyclins⁸⁻¹⁰. During the transition from the G₁ to the S phase of the cell cycle, Cdk's phosphorylate the Rb protein, changing its conformation and thus preventing it from binding to the E2F transcription factors¹. Since E2F proteins are involved in regulating cellular proliferation^{11,12}, mutation or loss of *Rb* often leads to aberrant proliferation and cancer in the affected organism. This was first recognized in humans with mutations in *Rb* who were prone to cancer of the retina, known as retinoblastoma^{3,13,14}. Defects in *Rb* can also manifest themselves in other tissue types. In mice, mutation or absence of *Rb* often manifests as cancer of the thyroid and the pituitary¹⁵.

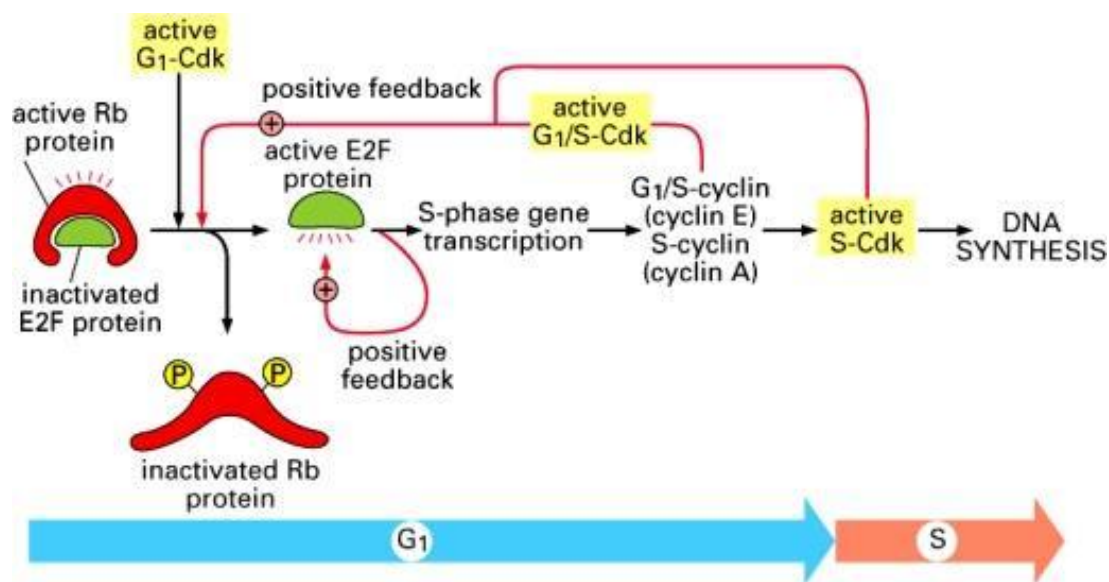


Figure 1⁴³. General Rb-E2F pathway whereby E2F activity is controlled by the phosphorylation state of Rb.

Developmentally, when both copies of *Rb* are non-functional, the mouse embryo dies around the embryonic age of 13.5 days¹⁶. This is thought to occur when E2F's that are no longer constricted by Rb bind to and activate genes necessary for progression past the G₁-S checkpoint. As this essentially nullifies the checkpoint, cells rapidly proliferate and cause developmental defects in the growing embryos. Specifically, in embryos lacking Rb, trophoblast stem cells in the placenta become over-proliferative. This overabundance of trophoblast cells disrupts the normal labyrinth structure of the placenta and severely disrupts the normal process of nutrient transport between the mother and the embryos¹⁶.

Analysis of the effect of the E2F family has been somewhat difficult, primarily due to differences in function among the various family members, as well as differences in tissue-specific functions. Currently there are 8 known E2F genes in the E2F family. It is thought that E2F1, E2F2, and E2F3a act as activators¹⁷, with their expression levels peaking during the G₁-S transition¹⁸⁻²¹. Conversely, E2F3b, E2F4, E2F5, E2F6, E2F7, and E2F8 are thought to act as repressors, allowing cells to exit the cell cycle and differentiate¹⁷ (**Figure 2**).

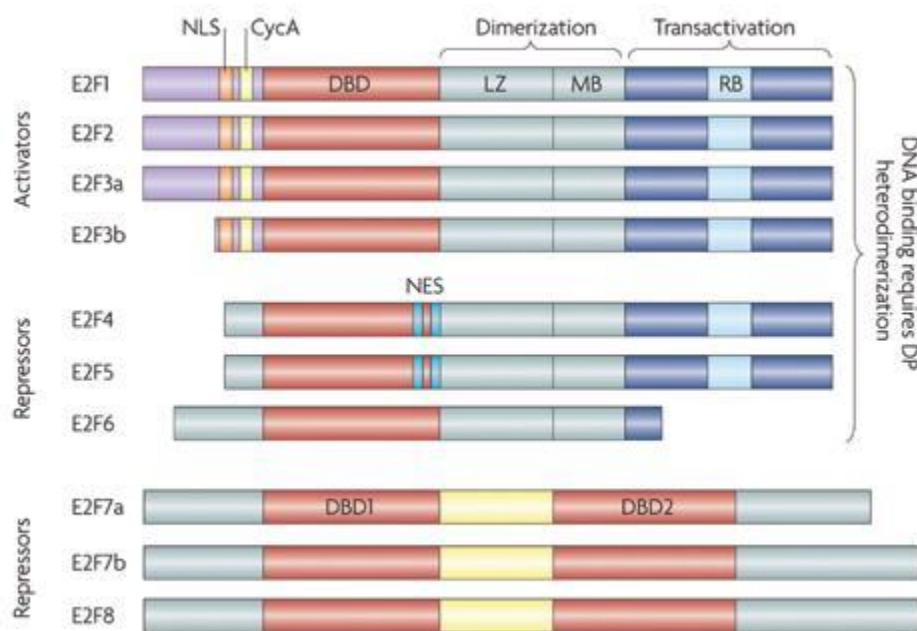


Figure 2¹⁷. Genetic loci of the E2F family members, including their functions as either activators or repressors of transcription, as well as functional domains of the protein.

The expression levels of E2F3b, 4, and 5 remain relatively constant during the cell cycle^{22,23} where they function to recruit Rb to E2F-target sequences and thus repress transcription at that site. This, however, is an oversimplified view of the issue. As mentioned earlier, the importance of the various E2F's appears to depend on the tissue. A lack of E2F1 and E2F2, for example, appears to cause diabetes in mice²⁴, showing a link between these specific E2F's and proper pancreas function. Furthermore, different members of the E2F family appear to have varying degrees of importance in certain biochemical processes and pathways. For example the *Myc* gene, a well known proto-oncogene, is known to induce S-phase as well as apoptosis. However, these effects are mitigated by specific members of the E2F family. When cells are deleted for E2F2 or E2F3, *Myc*'s ability to induce S phase is hindered; however, when deleted for E2F1 or E2F4 there are no noticeable differences²⁵. In a similar vein, *Myc*'s ability to induce apoptosis is reduced in cells deleted for E2F1, but not in cells deleted for E2F2 or E2F3²⁵. Such differences are often due to very slight physical changes between the proteins, such as the presence of a specific marked-box domain present in E2F1, but not in E2F3, which gives E2F1 its prevalence in the apoptosis pathway²⁶. It is also common for differences to be caused by tissue-specific upstream events that preferentially activate or repress certain members of the E2F family. In some cases, the specific E2F family member is not important, but rather its position on the chromosome determines how that locus is regulated spatiotemporally and thus determines the observed phenotype. For example, replacing exon 1a of E2F3 with exon 1b of E2F3 or the ORF of E2F1 suppresses phenotypes arising from loss of E2F3a²⁷. This points to transcriptional regulation of the given E2F3 locus, and not the specific form of the protein, as the major contributor to the observed phenotype.

This idea of specific functions within the subgroups of activators and repressors is

especially important for the process of proliferation itself and is one of the reasons why E2F3 is being focused on in this study. Previous work has shown that among the known activators, E2F3 appears to have a prominent role in regulating proliferation, more so than either E2F1 or E2F2 in some instances^{28,29}. A lack of E2F3 causes high levels of embryonic lethality in some mouse strains^{28,30,31}. However, a lack of either E2F1 or E2F2 does not seem to drastically affect development in mice. This observation was also supported by studies using mouse embryo fibroblasts (MEF's) in which deletion of E2F3 caused a marked decrease in proliferation, whereas deletion of either E2F1 or E2F2 showed a much milder decrease in proliferation³².

Another interesting observation is that activating members of the E2F family can often behave as tumor suppressors by inducing apoptosis in cells that are over-proliferative³³⁻³⁵. As mentioned earlier, mice heterozygous for the deletion of Rb die during embryonic development. One consequence of the Rb deletion is the induction of apoptosis via p53-dependent and independent pathways^{35,36}. Interestingly, deletion of E2F1 or E2F3 in Rb-null embryos suppresses this process of apoptosis²⁹. This suggests that these activating E2F's also have a role in promoting apoptosis. Lastly, in mice heterozygous for the Rb deletion, loss of E2F3 causes a decrease in pituitary tumors³⁶. It also causes an increase in medullary thyroid carcinomas with more aggressive metastases of these growths to other organs³⁶. One possible reason for the opposing effects seen in these organs could be tissue-specific differences present in either proliferative or apoptotic pathways. The natural levels and phosphorylation states of the various E2F's likely differs between the thyroid and the pituitary, accounting for the different trends observed.

This particular project was founded on a few key ideas collected from various sources in the literature. The first observation came from analyzing the E2F1 protein and how it interacts

with Rb. First, a number of potential phosphorylation sites were identified. Then, each of these sites were mutated to encode for alanine, thus preventing phosphorylation and creating a protein that is constitutively dephosphorylated. Two-dimensional gel analysis of wild-type E2F1 protein was performed, revealing two distinct spots for E2F1 on the gel. Only the mutation of serine to alanine at the 375th amino acid caused all E2F1 protein to migrate to only a single spot, representing the non-phosphorylated form of the protein³⁷. Rb-E2F1 binding assays have also been performed but the results are conflicting. One study, using *in vitro* assays, reported that E2F1 phosphorylation increased binding with Rb³⁷. Another study using *in vivo* assays, however, reported that E2F1 phosphorylation decreased binding with Rb³⁸. It seems, then, that E2F1 phosphorylation somehow affects binding with Rb and thus affects cell cycle regulation. We wanted to take this same concept and apply it to E2F3. The reasoning behind this was that E2F3 appears to have a more prominent role in proliferation than either E2F1 or E2F2. Sequence alignment between E2F1 and E2F3 was performed and the homologous phosphorylation site on E2F3 (amino acid 390) was found (**Figure 3.a**). This site was mutated to alanine, to mimic the non-phosphorylated form of E2F3, and was also mutated to aspartate to mimic phosphorylated E2F3. As in previous studies with E2F1, these E2F3 mutants share identical migration patterns with wild type E2F3 (**Figure 3.b**), providing evidence that these mutations are behaving as the phosphorylated and non-phosphorylated versions of the protein.

We introduced these same mutations in MEF's and performed proliferation assays to analyze which form of the protein most promoted or inhibited proliferation. MEF's with E2F3^{+/+}, E2F3^{SA/SA}, and E2F3^{SD/SD} mutations were examined for relative levels of proliferation under normal gene expression conditions. These results showed the highest level of proliferation for E2F3^{SD/SD} MEF's, followed by E2F3^{SA/SA} and then E2F3^{+/+}. However, another related

Materials and Methods

Creation of E2F3 mutant mice

Site-directed mutagenesis was first performed in bacterial cell lines. The mutation was made in exon seven of the E2F3 locus, creating a novel ScaI restriction site for the S390A mutation and a novel BamHI restriction site for the S390D mutation (**Figure 4.a**). Cutting of the DNA with these restriction enzymes allowed for initial confirmation of the mutations. DNA sequencing was also performed for confirmation (**Figure 4.b**). Embryonic stem cells (ES cells) were electroporated to transform the mutant DNA. A neomycin marker, flanked by loxP sites and near the mutation site, was used to select for ES cells containing the integrated mutant DNA. The ES cells were transplanted into a mouse blastocyst, creating a chimeric mouse which was then bred and further crossed to cre transgenic mice to remove the neomycin cassette. The initial presence and eventual removal of neomycin was confirmed by Southern blot and the genotypes confirmed by PCR (**Figure 4.c**). The mice were then bred into an FVB background and intercrossed with $Rb^{-/+}$ mice to obtain the desired genotypes.

Breeding strategy and harvesting of embryonic mice

$Rb^{+/-};E2F3^{+/SA}$, $Rb^{+/-};E2F3^{+/SD}$, $Rb^{+/-};E2F3^{SA/SA}$, and $Rb^{+/-};E2F3^{SD/SD}$ mice were crossed with other mice of identical genotype. The mothers were observed every morning for the presence of a vaginal plug, indicating a recent mating. This time point was considered E0.5 days. The embryos were harvested at the time points of E11.5 and E13.5 days. Prior to harvesting, the mothers were injected with BrdU (100 ug/g of body weight) interperitoneally. Heartbeats for each embryo were observed under a microscope as an indicator of survival. The embryos and their placentas were immediately fixed in formalin for histological analysis. Yolk sacs were collected to genotype the embryos. The placentas were stained with haematoxylin and

eosin to look for potential differences in cellular crowding in the placental labyrinth structures.

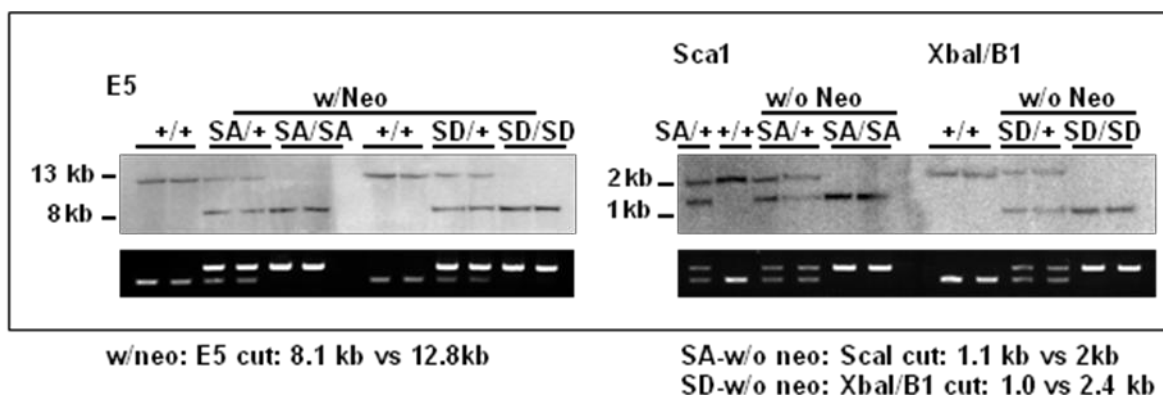
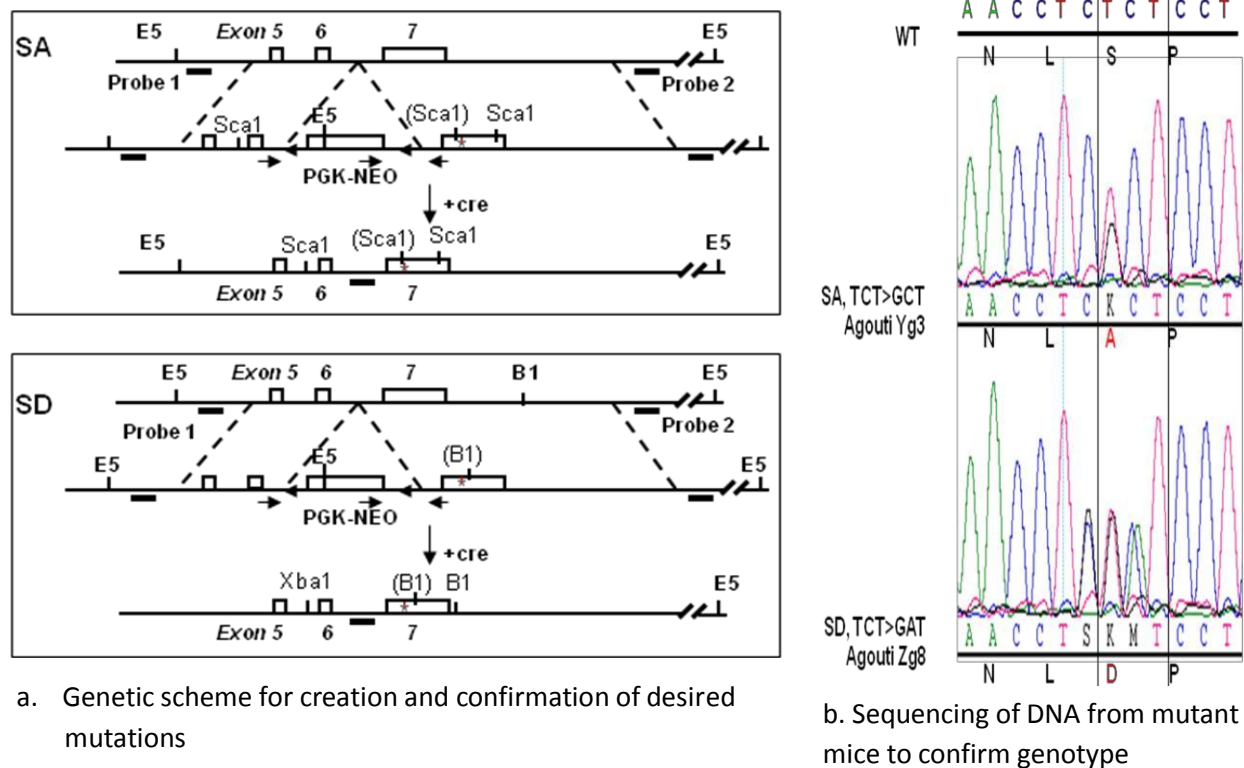
PCR

DNA was purified from tail samples or embryonic yolk sacs. Standard PCR was used to amplify the desired fragments. Primers used for E2F3 genotyping were as follows:

AAAGCTCTTGGGAAGTTCAG and GGGAGGACACAGATCACCAT. Primers used for Rb genotyping were as follows: GAAGAACGAGATCAGCAG,

ACAGGATGACTCACGGTACCTC, and CTGCTGGGATCAGTTAATGCTT.

Figure 4. (Work and images by Hui Wang, Ph.D.)



c. Confirmation by southern of pre-neomycin deletion genotypes (top left) and of mice genotypes after cre-induced neomycin deletion (top right). Confirmation by PCR for pre-neomycin deletion (bottom left) and for mice after cre-induced neomycin deletion (bottom right)

Harvest and tissue collection of adult mice

Mice were bred to obtain $Rb^{+/-};E2F3^{SA/SA}$, $Rb^{+/-};E2F3^{+/+}$, and $Rb^{+/-};E2F3^{SD/SD}$ genotypes. These mice were harvested at 11 months. We harvested the pituitary, thyroid, lung, liver, kidneys, adrenal glands, pancreas, bladder, mammary glands, salivary glands, and femur. Pictures were taken of the pituitaries and of any other abnormalities noticed in the organs. The tissues were immediately fixed in formalin. Large tumors of the thyroid and pituitary were taken for both histology and for protein samples.

Results

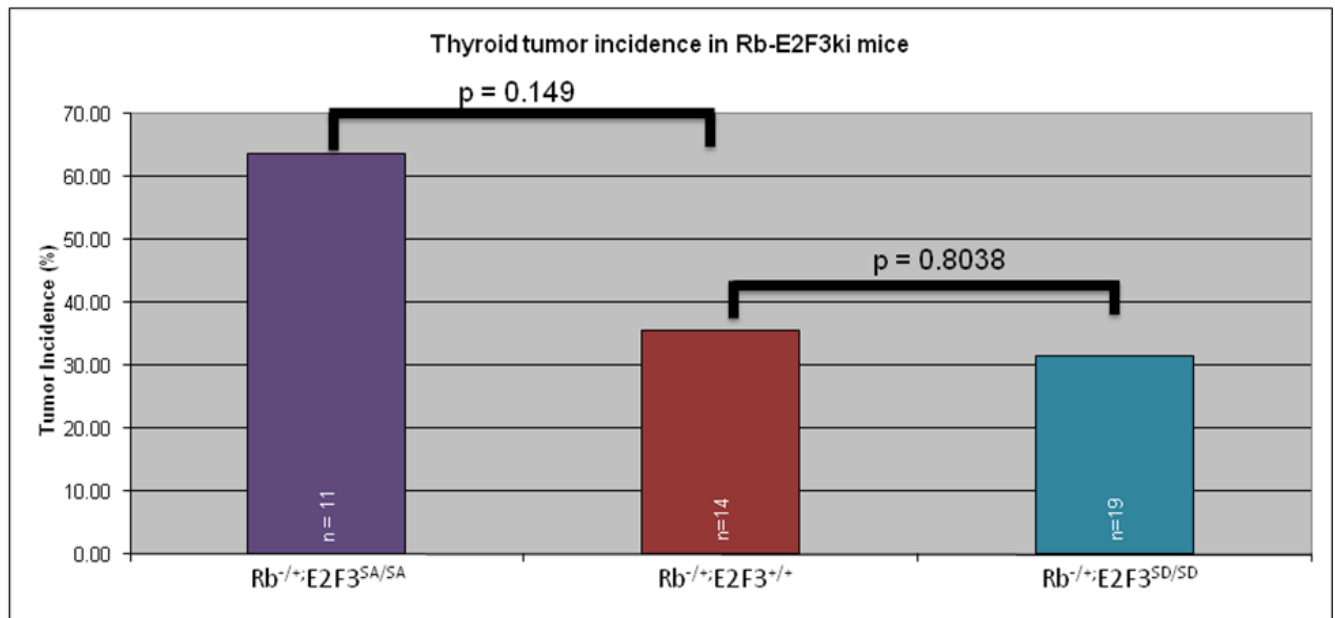
Effect of E2F3 phosphorylation in Rb-mediated tumorigenesis

Previous work was done on $Rb^{-/+};E2F3^{-/-}$ mice in which deletion of E2F3, when compared to wild type, caused a decrease in pituitary tumors but an increase in medullary thyroid carcinomas, accompanied by metastases to other organs such as the lung and the liver³⁶. In that study, E2F3 had zero functionality within the organism. We wanted to assay whether the S390A or S390D mutation would have a significant effect on the protein's functionality and thus mimic the knockout. We used $Rb^{-/+};E2F3^{+/+}$ mice as a control and compared them to $Rb^{-/+};E2F3^{SA/SA}$ and $Rb^{-/+};E2F3^{SD/SD}$ mice. Previous studies showed that $Rb^{-/+}$ mice lived to approximately 10 months of age. We noted a slightly extended lifespan and thus harvested the mice at 11 months of age. One result of this study was that $Rb^{-/+};E2F3^{SA/SA}$ mice seemed to have a higher incidence of thyroid growths than either $Rb^{-/+};E2F3^{+/+}$ or $Rb^{-/+};E2F3^{SD/SD}$ (**Figure 5.a**). Within the $Rb^{-/+};E2F3^{SA/SA}$ genotype, female mice showed a significantly higher incidence of thyroid cancer than males compared to the other groups (**Figure 5.b**).

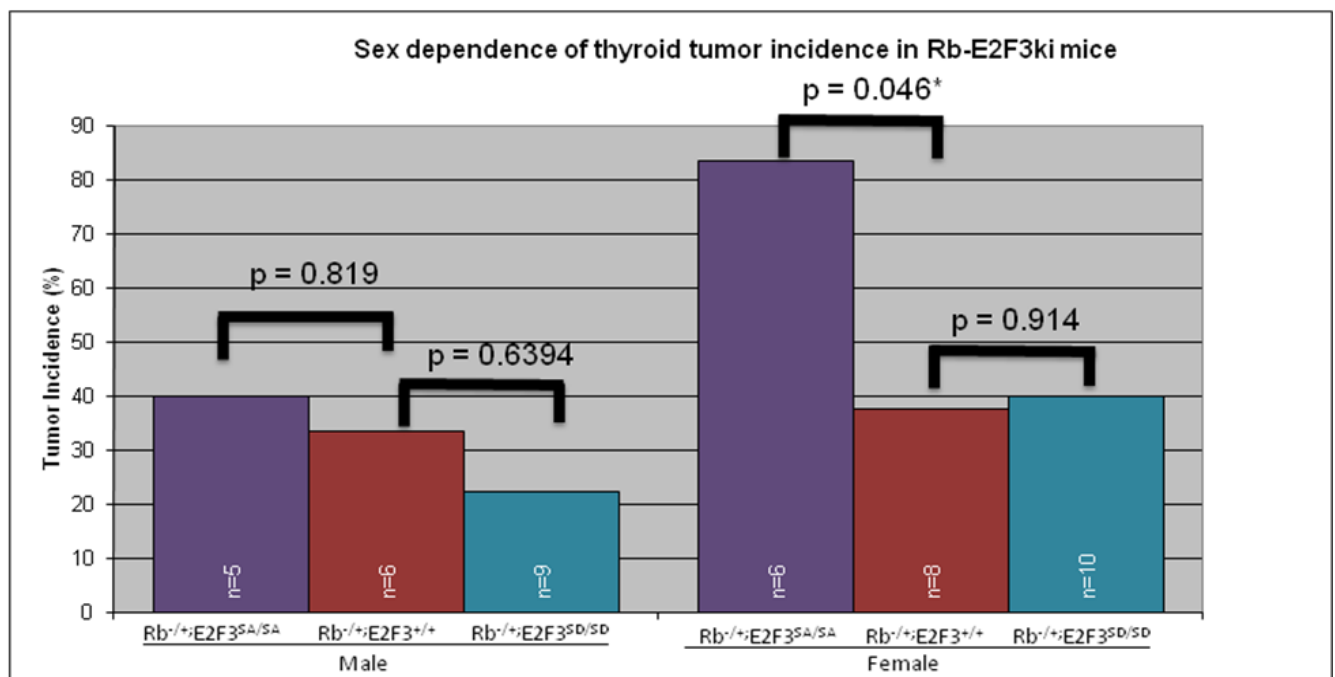
Analysis of the pituitaries also showed an interesting trend. Unfortunately histological analysis of the pituitaries was not possible and thus the following observations are based on

gross analysis of the pituitaries. Tumor incidence seems relatively similar among $Rb^{-/+};E2F3^{+/+}$, $Rb^{-/+};E2F3^{SA/SA}$ and $Rb^{-/+};E2F3^{SD/SD}$ mice (**Figure 6.a and b**). However, tumor size appears to be greatest in the control mice. (**Figure 6.c**).

Figure 5.

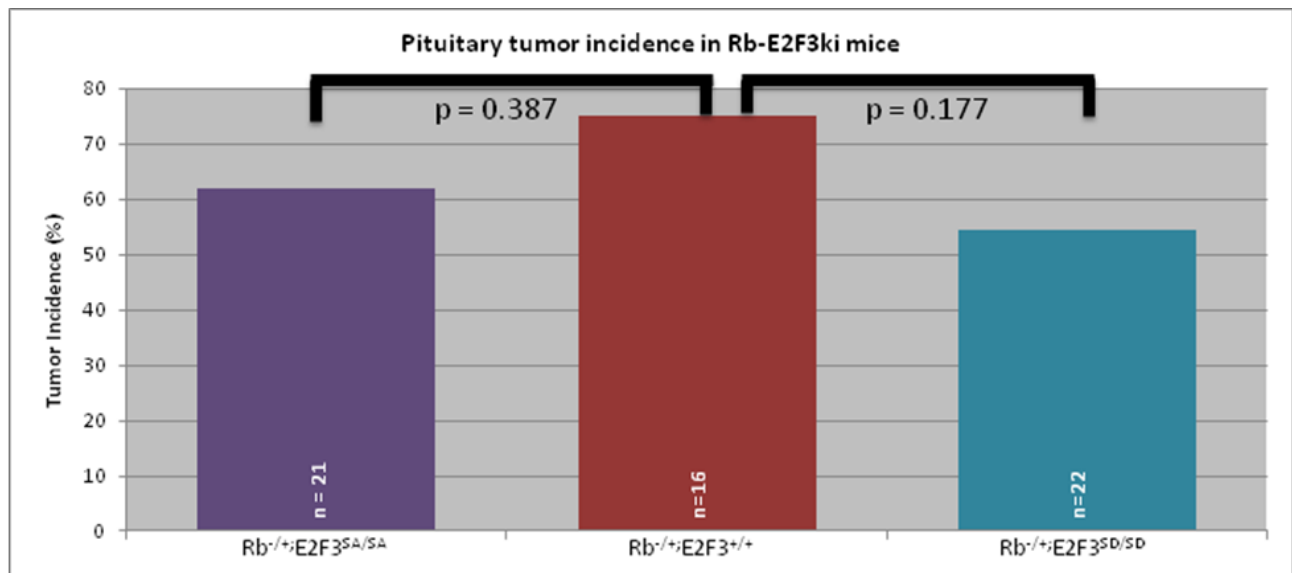


- a. $Rb^{-/+};E2F3^{SA/SA}$ appear to have a higher incidence of thyroid growths than either $Rb^{-/+};E2F3^{SD/SD}$ or $Rb^{-/+};E2F3^{+/+}$

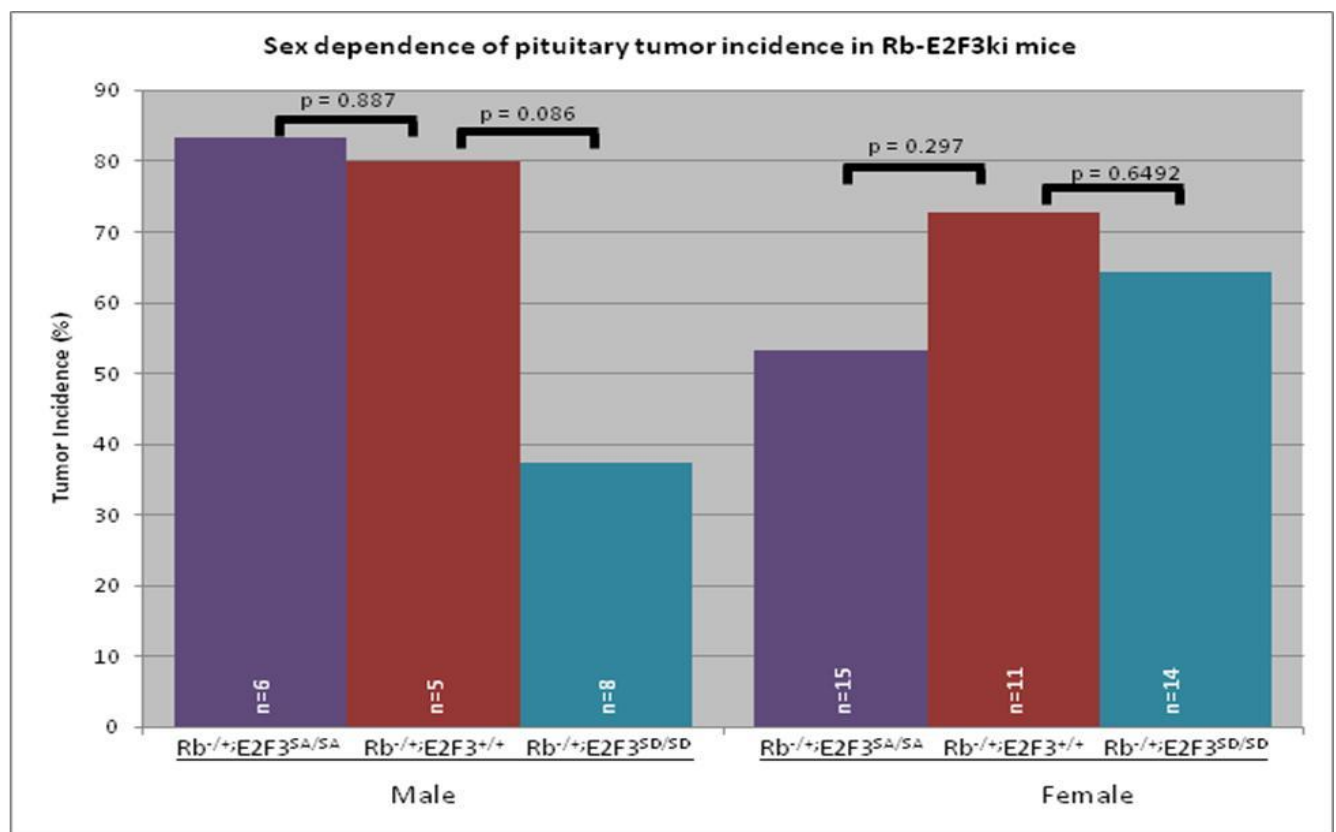


- b. $Rb^{-/+};E2F3^{SA/SA}$ female mice, but not male mice, show an increase in thyroid cancer compared to either $Rb^{-/+};E2F3^{SD/SD}$ or $Rb^{-/+};E2F3^{+/+}$. This suggests a sex-dependent mechanism of tumorigenesis in the thyroid, which is also observed in human females. (* indicates a significant difference is present)

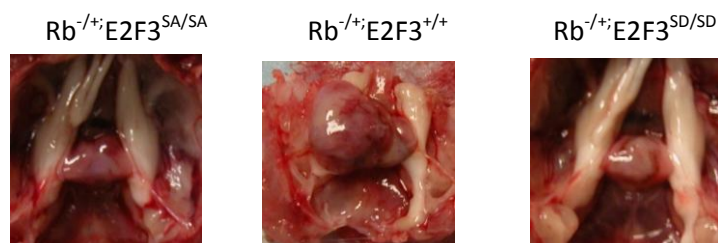
Figure 6



a. Graph showing no apparent differences in pituitary tumor incidence between any of the genetic groups.



b. Graph showing no apparent sex-dependent differences in pituitary tumor incidence between any of the genetic groups.



c. It was generally observed that pituitary size was surprisingly most severe in the Rb^{-/-};E2F3^{+/+} mice.

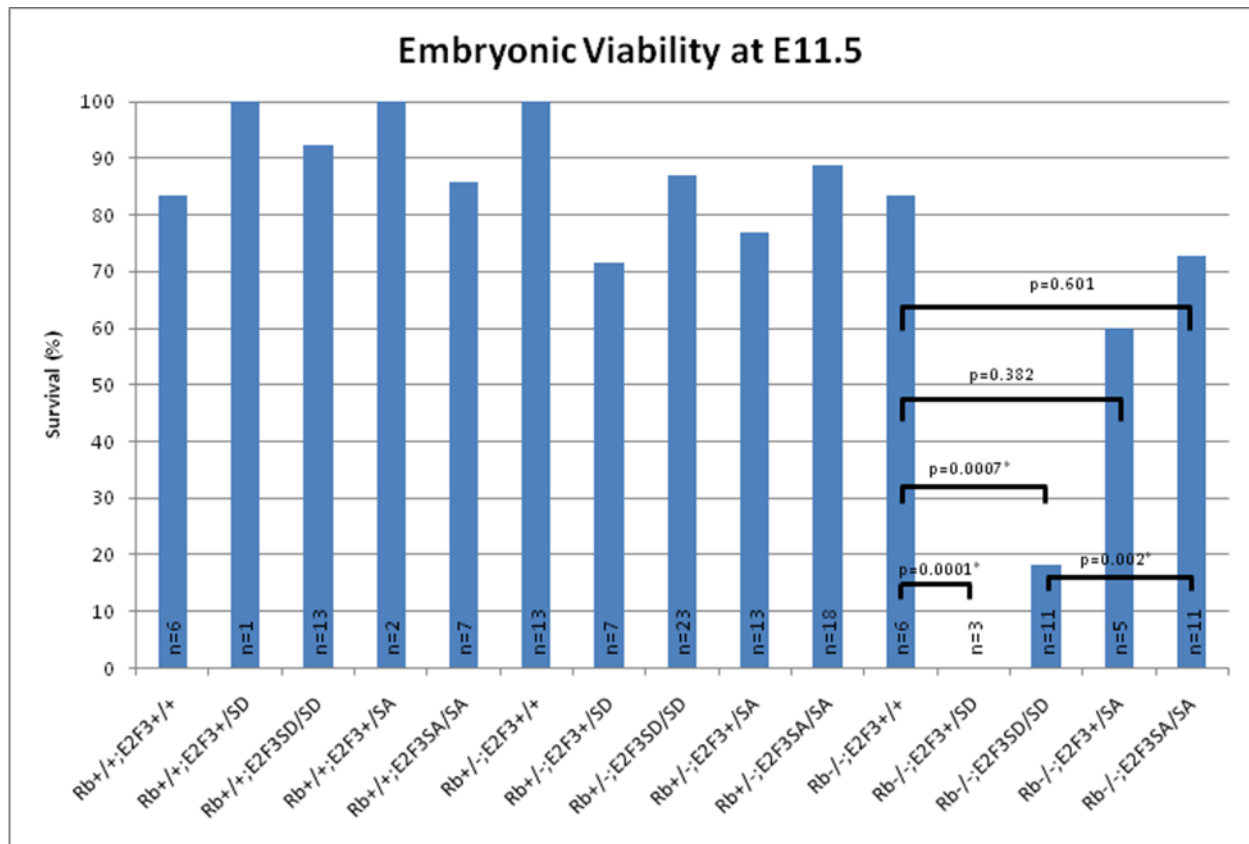
Effect of E2F3 phosphorylation on embryonic development in Rb-null mice

It has been shown that embryos with both copies of Rb knocked out fail to develop normally. These embryos show ectopic apoptosis and proliferation in the central and peripheral nervous system, in the placenta, and in other organs. Furthermore, erythroid, neuronal, and skeletal muscles cells do not terminally differentiate^{15,39,40}. It is thought that a combination of erythroid abnormalities and placental abnormalities cause fetal death by the embryonic age of 13.5 days. Previous studies have shown that by knocking out E2F3 in these Rb-null embryos, some of these defects are alleviated, such as an increase in proper enucleation of red blood cells³⁵. Furthermore, the lifespan of these embryos increases, although they still do not survive to term due to the severity of the Rb-null phenotype. We first observed embryos at E13.5. In the control group, Rb^{-/-};E2F3^{+/+} mice, only 2/17 (~12%) mice were found alive. 2/15 (~13%) Rb^{-/-};E2F3^{+/SD} mice and 0/6 (0%) of Rb^{-/-};E2F3^{SD/SD} were found alive at this point (**Figure 8**). The survival for embryos with the S390A mutation was slightly higher, although not by a significant amount. 3/13 (23%) of the Rb^{-/-};E2F3^{+/SA} mice and 1/6 (17%) of the Rb^{-/-};E2F3^{SA/SA} mice were found alive at this point. It seems, then, that the Rb mutation is dominant by this point in development and these mice are dying regardless of the state of E2F3. We then observed the embryos at E11.5 and noticed a trend that was not seen before. In the control group, Rb^{-/-};E2F3^{+/+}, 5/6 (83%) of the embryos were still alive at this age (**Figure 7**). 0/3 (0%) Rb^{-/-};E2F3^{+/SD} mice and 2/11 (18%) of Rb^{-/-};E2F3^{SD/SD} were found alive at this point. Interestingly, the survival for S390A embryos was much higher than S390D embryos at this point. 3/5 (60%) Rb^{-/-};E2F3^{+/SA} mice and 8/11 (73%) of Rb^{-/-};E2F3^{SA/SA} were found.

Previous studies also show that one of the primary causes of embryonic lethality due to a lack of Rb is overproliferation in the trophoblast of the placenta¹⁶. This limits nutrient transport

between the mother and fetus and eventually causes death. Comparison of the hematoxylin and eosin stained placentas produced observations which correlated with the experimental findings. The placentas from E11.5 $Rb^{-/-};E2F3^{SD/SD}$ embryos had fewer maternal blood spaces and an overall greater degree of cellular crowding than either $Rb^{-/-};E2F3^{SA/SA}$ or $Rb^{-/-};E2F3^{+/+}$ (**Figure 9**). This points to a similar mechanism presented in previous work where the E2F3 induced proliferation was the primary cause of death. Ablation of E2F3 function in the cell increased the lifespan of these organisms, just as may have occurred in this experiment.

Figure 7.



- a. At E11.5 the vast majority of $Rb^{-/-};E2F3^{SD/SD}$ and $Rb^{-/-};E2F3^{SD/+}$ mice were dead. However, Rb -null embryos with wild-type E2F3 or the SA mutation in any number of copies did not vary significantly and were primarily alive at this time point. (* indicates a significant difference is present)

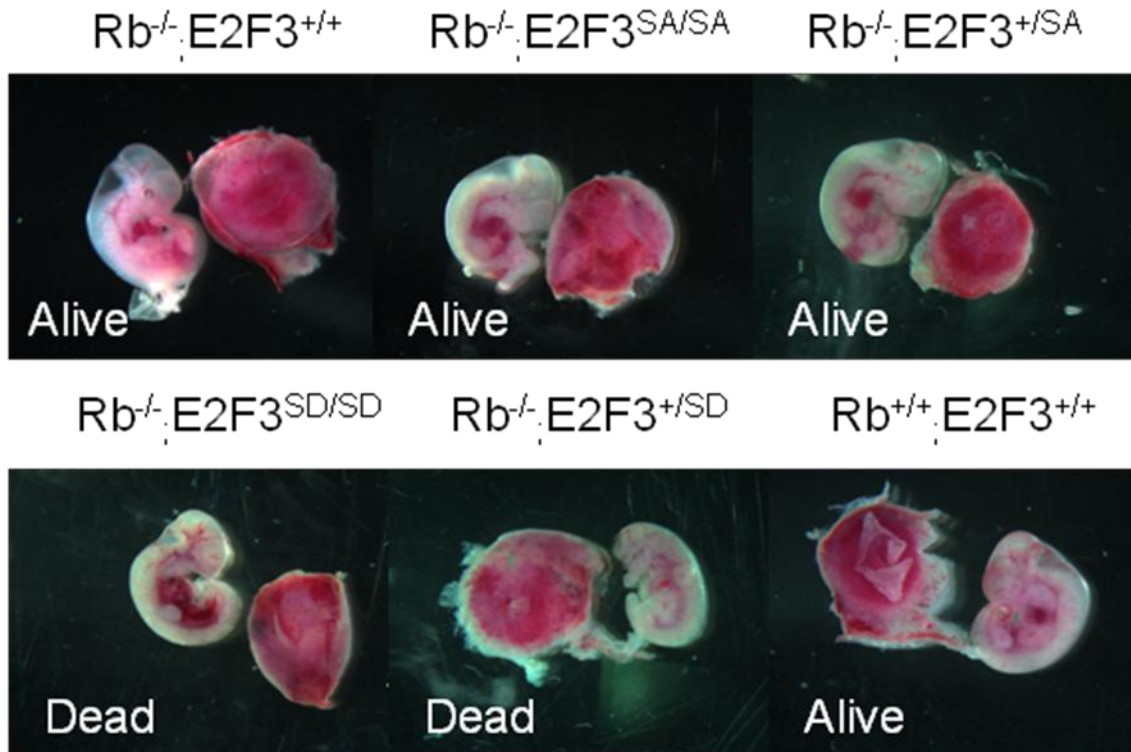
Format: # Alive(#Dead)

SD	E11.5										
	Rb ^{+/+} ; E2F3 ^{+/+}	Rb ^{+/+} ; E2F3 ^{+/-SD}	Rb ^{+/+} ; E2F3 ^{SD/SD}	Rb ^{+/-} ; E2F3 ^{+/+}	Rb ^{+/-} ; E2F3 ^{+/-SD}	Rb ^{+/-} ; E2F3 ^{SD/SD}	Rb ^{-/-} ; E2F3 ^{+/+}	Rb ^{-/-} ; E2F3 ^{+/-SD}	Rb ^{-/-} ; E2F3 ^{SD/SD}	Total	Avg litter ize
Observed	1(1)	1(0)	12(1)	3(0)	5(2)	20(3)	2(0)	0(3)	2(9)	46(19)	9.29 pups
Expected	2	3	12	3	6	23	2	3	12	66	

SA	E11.5										
	Rb ^{+/+} ; E2F3 ^{+/+}	Rb ^{+/+} ; E2F3 ^{+/SA}	Rb ^{+/+} ; E2F3 ^{SA/SA}	Rb ^{+/-} ; E2F3 ^{+/+}	Rb ^{+/-} ; E2F3 ^{+/SA}	Rb ^{+/-} ; E2F3 ^{SA/SA}	Rb ^{-/-} ; E2F3 ^{+/+}	Rb ^{-/-} ; E2F3 ^{+/SA}	Rb ^{-/-} ; E2F3 ^{SA/SA}	Total	Avg litter size
Observed	4(0)	2(0)	6(1)	10(0)	10(3)	16(2)	3(1)	3(2)	8(3)	62(12)	9.25 pups
Expected	3	6	10	6	12	20	3	6	10	74	

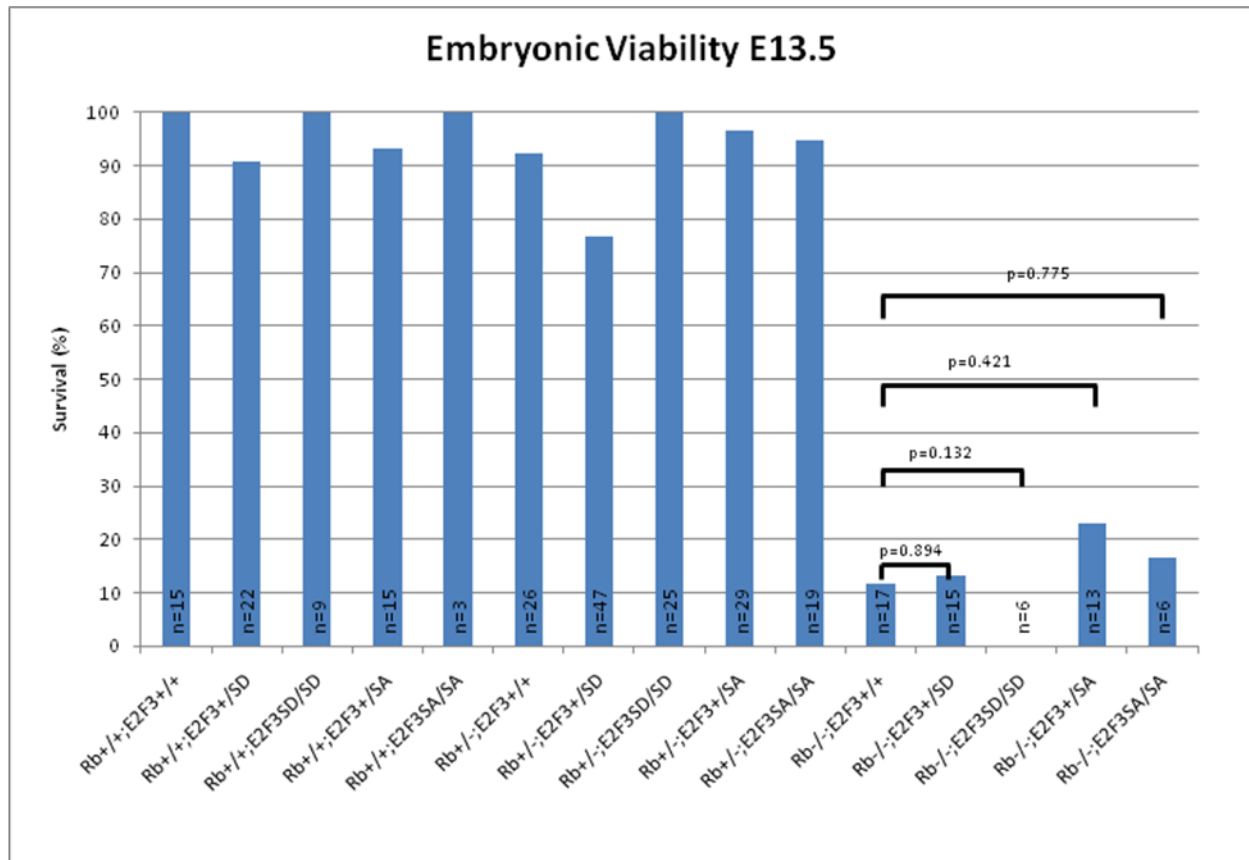
- b. Table showing the exact values obtained for the survival of embryos at E11.5. Also shows the expected mendelian ratios from the crosses performed.

E11.5



- c. Pictures of typical embryos at E11.5. Death of embryos noted primarily by presence or absence of heartbeat. Pale color and hemorrhaging also acted as additional indicators of embryonic death.

Figure 8.



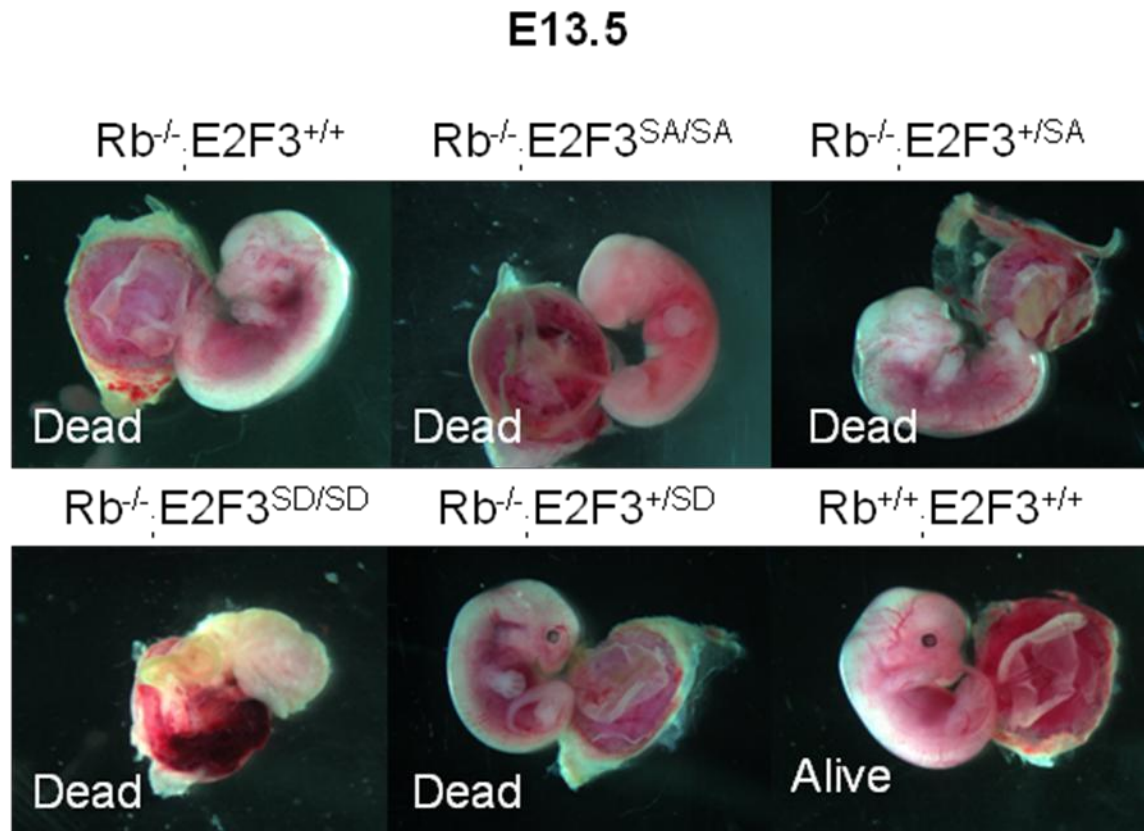
- a. At E13.5 the vast majority of all Rb-null mice were dead. The lack of Rb had become the dominating phenotype and appears to be masking any phenotypes we might have observed from E2F3. (* indicates a significant difference is present)

Format: # Alive(#Dead)

SD	E13.5										
	Rb ^{+/+} ; E2F3 ^{+/+}	Rb ^{+/+} ; E2F3 ^{+/-} /SD	Rb ^{+/+} ; E2F3 ^{SD/SD}	Rb ^{+/-} ; E2F3 ^{+/+}	Rb ^{+/-} ; E2F3 ^{+/-} /SD	Rb ^{+/-} ; E2F3 ^{SD/SD}	Rb ^{-/-} ; E2F3 ^{+/+}	Rb ^{-/-} ; E2F3 ^{+/-} /SD	Rb ^{-/-} ; E2F3 ^{SD/SD}	Total	Avg litter size
Observed	9(0)	20(2)	9(0)	12(0)	36(11)	25(0)	2(11)	2(13)	0(6)	115(43)	9.29 pups
Expected	10	20	10	20	39	20	10	20	10	159	

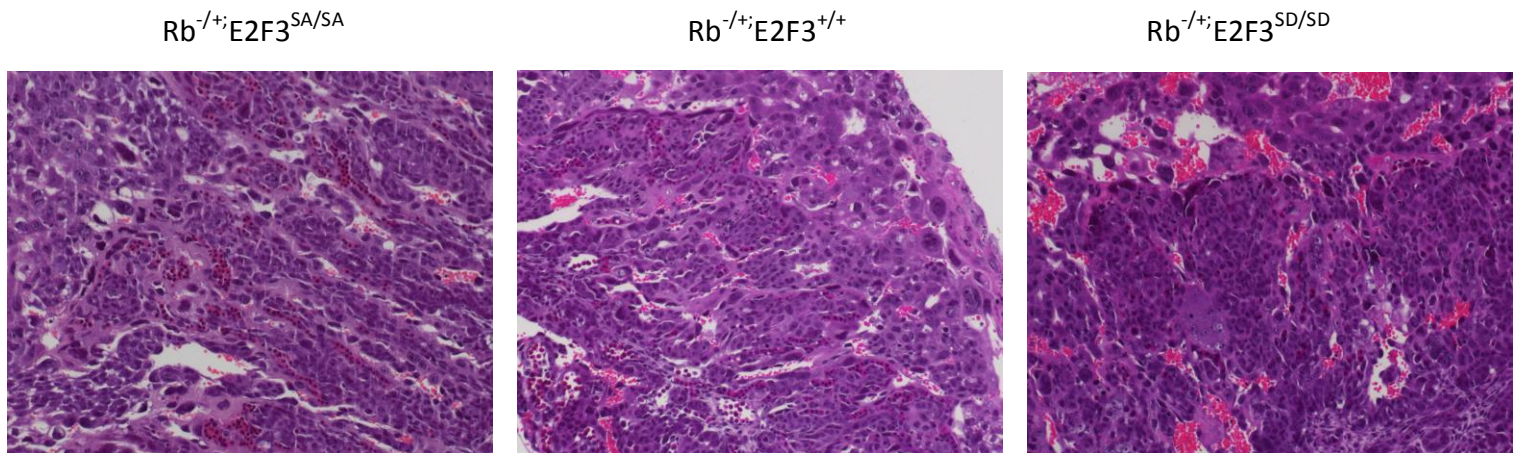
SA	E13.5										
	Rb ^{+/+} ; E2F3 ^{+/+}	Rb ^{+/+} ; E2F3 ^{+/SA}	Rb ^{+/+} ; E2F3 ^{SA/SA}	Rb ^{+/-} ; E2F3 ^{+/+}	Rb ^{+/-} ; E2F3 ^{+/SA}	Rb ^{+/-} ; E2F3 ^{SA/SA}	Rb ^{-/-} ; E2F3 ^{+/+}	Rb ^{-/-} ; E2F3 ^{+/SA}	Rb ^{-/-} ; E2F3 ^{SA/SA}	Total	Avg litter size
Observed	6(0)	14(1)	3(0)	12(2)	28(1)	18(1)	0(4)	3(10)	1(5)	85(24)	8.38 pups
Expected	7	14	7	14	27	14	7	14	7	111	

- b. Table showing the exact values obtained for the survival of embryos at E11.5. Also shows the expected mendelian ratios from the crosses performed.



- c. Pictures of typical embryos at E13.5. Death of embryos noted primarily by presence or absence of heartbeat. Pale color and hemorrhaging also acted as additional indicators of embryonic death. Both higher mortality and a greater degree of reabsorption were noted at E13.5 compared to E11.5

Figure 8.



H&E staining of E11.5 placentas showing a greater degree of cellular crowding in the labyrinth of $Rb^{-/+}; E2F3^{SD/SD+}$ placentas (right) that is potentially causing earlier lethality due to lack of correct nutrient transport between mother and pup.

Discussion

This study sought to analyze the effects of E2F3 phosphorylation *in vivo* in the absence or partial absence of the *Rb* gene. Previous work demonstrated a role for E2F3 in both tumorigenesis as well as embryonic development. That work, however, was only performed by knocking E2F3 out of the organisms. Phosphorylation, conversely, is a more biologically relevant and common change found to affect the way that proteins function and interact with each other. With this in mind, we hoped to understand how this phosphorylation affects E2F3's activity in the cell and in the context of cancer. Ultimately, we hope to understand this mechanism in order to create novel chemotherapies for cancers propagated in this manner.

To reiterate, one main finding of this study so far is that the E2F3^{S390A} mutation (non-phosphorylation mimicking) appears to cause an increase in thyroid tumor incidence, primarily in females. Interestingly, both E2F3^{S390A} and E2F3^{S390D} appear to decrease average pituitary tumor size, although this data has not been quantified. Another finding is that the *Rb*^{-/-};E2F3^{S390D} mutation causes earlier embryonic lethality than either *Rb*^{-/-};E2F3^{+/+} or *Rb*^{-/-};E2F3^{S390A}.

The reasons behind these trends are still unclear, although a few models have been suggested. E2F1 and E2F3 are known to share similar functions. Phosphorylation has not been well studied in E2F3; however, there have been a number of studies of phosphorylation at various sites in E2F1. It is likely that E2F1 and E2F3 function in a somewhat similar manner. Evidence for this comes from their seemingly overlapping abilities to support proliferation as well as development when either of the E2F's is knocked out. One general mode of action that has been suggested is as follows: when Rb is phosphorylated, it releases E2F1 which proceeds to bind to its target DNA. This binding activates the transcription of genes, including

genes necessary for replication, which code for cyclins, cdk's, and other necessary proteins.

Two of these targets, which are also a targets of E2F3, are cyclin A and cdk2. The E2F1 protein contains a binding site for cyclin A which then allows the attached cdk2 to associate with and phosphorylate E2F1 (as well its heterodimeric partner DP-1) at serine 375. This phosphorylation has been found to weaken E2F1's binding to DNA, as well as strengthen its binding to Rb³⁷.

This then acts as a negative feedback loop, ensuring that E2F's do not promote active transcription of replication-inducing genes for too long. Applying this same model to the results obtained gives somewhat mixed results. This could help to explain why thyroid tumor incidence is more prevalent in the E2F3^{S390A} mice, but doesn't help to explain the gender bias observed. Furthermore, it does not explain why mice wild-type for E2F3 show more severe pituitary tumors or why embryonic lethality would occur earlier for E2F3^{S390D} mice. E2F1 and E2F3, while very similar, still have differences both in terms of protein structure and expression patterns. Perhaps, then, phosphorylation does not affect Rb-binding affinity similarly between these two proteins. It's possible that phosphorylated E2F3 binds more tightly to DNA and is thus why proliferation occurs more rapidly in these cells. This would explain the presence of phosphorylated E2F3 in mammary tumor cell lines, as well as the increased levels of proliferation noted in the E2F3^{S390D} MEF's. This mode of operation, however, then contradicts the results found in the pituitary and suggests that a more complex level of regulation and activity is present.

Previous work has shown that E2F1-3 can behave as activators under some conditions, but repressors under others⁴². This brings to light the possibility that such differences could also be present in a tissue-specific manner. It is possible that the phosphorylated forms of E2F3 could be behaving as activators in one scenario, and repressors in another, giving the somewhat

conflicting phenotypes observed in this study. Other complexities could arise from the fact that E2F3 is known to signal for apoptosis in over-proliferating cells³⁵. Apoptosis signaling is another biochemical pathway known to vary from tissue to tissue, and could be one reason why tumor spectrums vary across the genotypes. Yet another source of complexity comes from differential activation of tissue-specific genes depending on which E2F is present. For example, PTTG-1, which is primarily activated by E2F1, is a gene that when overexpressed leads to cancer of the pituitary, thyroid, and other endocrine glands, with other tissues being unaffected⁴¹. This is merely one example but shows that tissue specific gene induction also plays a role in tumorigenesis in an E2F dependent manner.

For future experiments we would like to analyze proliferation as well as apoptosis in the pituitary and thyroid. Furthermore, in $Rb^{-/+};E2F3^{-/-}$ experiments, mice showed an increase in metastases from the thyroid to the lung, liver, and other organs. We wish to quantify the level of c-cell invasion in these tissues to ascertain whether a similar trend exists with phosphorylation. We would also like to look into phosphorylation-specific effects on Rb-E2F3 binding. The strength with which each of these mutant E2F3's binds to Rb could give us insight into how E2F3 and Rb are interacting *in vivo* and whether this behavior is similar to or different than E2F1. Hopefully by understanding E2F3's behavior *in vivo* we will eventually be able to regulate it to help prevent a large variety of cancers.

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